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**ADVANCES IN THE DIAGNOSIS OF BRUCELLOSIS
IN MAN AND OTHER ANIMALS**

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(With the authorization of the author and through the courtesy of Dr. Clemente Carrillo Cardenas, who translated this article into Spanish from English, as originally published by the World Health Organization, Joint FAO/WHO expert Committee on Brucellosis, Geneva, 3-9 December 1963, Agenda Item 5, we gave permission to reproduce this article because of the importance of the topic discussed in it and because the author mentions experimentation material provided by the Medical Research Institute of the General Hospital SSA.)

We have little progress in the laboratory diagnosis of brucellosis in man and other animals since the last meeting of the committee of experts in brucellosis of the FAO/WHO (1957) and the publication of Castaneda (1961). Ramacciotti (1958) wrote a book on this subject.

A number of promising tests have not yet been sufficiently thoroughly studied and examined for recommendation and use.

1. First Isolation and Subsequent Cultivation of Brucella

The isolation of brucella from the patient and other animals, or their excretions, is still the one laboratory test which we have the most confidence in when it comes to the diagnosis of brucellosis.

(a) Hemoculture

The double cultivation medium of Castaneda (1947, 1957) is still the most important method here in hemoculture. The same can be said about heat and about agar albini and the "tripticase" [tryptic] soy bean as the best media for the isolation and growth of brucella.

Agar with sugar and dextrose produces good results as a culture medium and can be used when the dehydrated medium is not satisfactory. The egg, as a cultivation medium, appears to improve the yield of brucella from blood, as described by Calderone and Pickett (1961).

Concentration methods, such as the filtration method (Braun and Kelsch, 1954; Reusse and Schindler, 1957), the erythrocyte lysis method (Pickett and Nelson, 1951) and others have not been used much.

Huddleston (1957, 1959) described a rather interesting technique for obtaining hemocultures. It is based on the growth of brucella in human defibrinated citrated blood, although cow and horse blood can also be used, in the presence of the exchange of the hydrogen ion of the resins (Duolite C-3H + y Duolite C-3Mg ++). We mix 10 cc of blood directly with the sterile dilution in the autoclave [with the] resin [diluted] to 10% of the citrate of sodium; the sample may be kept in a suitable agar cultivation medium at 37° C for 24, 48 and 72 hours in incubation.

Broader experimental and comparative studies are necessary in order to confirm the usefulness of this method for the customary isolation of bacteria from the blood of patients, as recommended by Huddleston (1961).

(b) Cultivation with Milk and Other Materials

Because of the presence of various species of bacteria in milk and in other specimens [samples], it is recommended that we use antibiotics in the culture media in our routine work.

The cultivation medium of Kuzdas and Morse (1953) and the modification of this medium by Renoux (1954) are the best for this purpose. Circulin, included in both cultivation media, is not very necessary and may be omitted from the formula.

The brucella may be isolated most easily from the cream of cow's milk, the cream and sediments of goat's milk after running it through the centrifuge. The isolation of cow's

milk can be improved by filtering it (Reusse, 1959).

(c) Inoculation of the Animal

The culture media used so far, primarily those that contain antibiotics, are so efficient for the first-isolation of brucella that it is not necessary to use laboratory animals for this purpose. However, if necessary the laboratory animal indicated here would be the guinea pig; if no guinea pigs are available, we can even use white rats.

Inoculation in chicken eggs with embryo is not very recommended in spite of what some investigators have said (Ramacciotti, 1958).

(d) Subsequent Cultivation of Isolated Brucella and Their Storage

The Albimi, the "Trypticase" soy bean and agar serum dextrose appear to be the best for the subsequent cultivation of the isolated brucella. This recommendation, contained in previous reports by the committee of experts on brucellosis of the FAO/WHO, for the purpose of avoiding subcultures in liquid media, must be taken into consideration as a point of concern in the routine to be employed so as to prevent the appearance of variants which are not neat and clean and definite.

The cultures can be kept in a refrigerator in well-taped closed tubes. However, the best way of keeping brucella cultures and preserving the desired characteristics in them is lyophilization. The dry and cold frozen cultures should be kept in the refrigerator at a temperature of 4° C.

2. Serological Tests

We have been able to observe a number of advances in laboratory and field serological tests.

(a) Agglutination Tests

The method of dilution in the tube is recommended as the only procedure worthy of confidence in the diagnosis of the individual cases. The value of this method is confirmed by the abundant literature published after the 1957 meeting of the committee of experts on brucellosis of the FAO/WHO.

The agglutination test, performed with antigen, stained with chloral hydrate, 2, 3, 5-triphenyltetrazolium, as suggested by Mello (1951), Renoux (1952), and Aneczykoski (1958) enables

us to differentiate the agglutinated brucella of the nonspecific sediments ~~/the brucella agglutinated by the nonspecific sediments/~~ and also makes it easier to read the final titer. (For further information and technical details, see Anoykowski, 1958, 1959).

The rapid plate method, used for the diagnosis of animal brucellosis in many countries throughout the world, requires much care. The slightest mistake may lead to tremendous errors in the interpretation of the tests. The results obtained by Pope and Ruedy (1959) are important because they show that high environmental temperatures increase the titer in ~~/by/~~ the number of suspect reactors. This is very important in tropical areas where the tests are performed in the open air and at relatively high environmental temperatures.

Many investigators have for quite some time been describing the agglutination test with whole blood; but it might perhaps be a good idea to subject this test to new comparative studies which would enable us to arrive at a convenient standardization.

It is possible that this test might eventually become the best among all of the field tests to be performed among vast population groups. Recently, Castaneda (1961) reported the method for the preparation of the antigen and the verification of the same which he called the spot test (Castaneda, 1953). The test has been used for more than 20 years by Mexican laboratories; Spink and Anderson (1952) tried to standardize it in order to obtain positive tests only when the agglutinins exist in quantities of 1:100 or more. In spite of this, this test has not been met with general acceptance. The test has been used in many parts of the world ever since 1935 (Welch and Marsh, 1935) and was recommended for a rapid diagnosis of animal brucellosis in the field; it seems to offer some real advantages for rapid use, especially in slaughterhouses.

The details of the preparation of the antigen and the development of the test can be found in the publications of Boulanger and Smith (1957) and Boulanger et al (1958) in Canada, and Cedro et al (1953, 1955, 1960) of Argentina.

In differentiating between the specific or nonspecific agglutination reactions, it seems that the best way to inhibit nonspecific reactions is to inactivate the serum by means of heat at a temperature of 65° C for 15 minutes. The case was reviewed by Amorault et al (1961); according to the authors, the test is good for cattle serum with suspect titers; in the majority of the infected animals, even when their agglutination

reactions are weak, we can discover the infection in this manner. In a recent communication, Lambert and Amerault (1962) reported that the tests with serum, rendered inactive by heat, are more effective than the complement fixation reaction in discovering a recent infection and in clarifying the state of some animals treated with a sero-agglutination test and in those in which we have reason to suspect brucellosis.

(b) Complement Fixation /Reaction/ Test (C.F.)

This test was one of the first to be used in the diagnosis of brucellosis in man and other animals. In spite of this, it has not yet become standardized. It is recommended for diagnosis in individual cases. The study by Foz (1953) with the serum of 197 people showed that C.F. is specifically recommendable. In patients who have had brucellosis for a few days or weeks, C.F. can be negative even though the sero-agglutination test may be positive; in cases of chronic brucellosis (lasting more than 6 months), the titer of the C.F. is higher than that of sero-agglutination; in cases of cured or long-lasting brucellosis, the C.F. may be positive while sero-agglutination may be negative.

These results, in relation to the duration of the disease as such, were not confirmed by Gargani (1959) who, for purposes of comparison, worked with sero-agglutination and C.F. in 347 human sera. (Results positive in both, 70; results positive only with sero-agglutination, 34; results positive with C.F. only 6).

Gargani and Aleandri (1959) reviewed this case. On the other hand, reviewing the most important literature on the subject, the details emerge quite clearly with respect to the procedure and the results of the examination of 347 human sera mentioned above.

The authors recommend the presentation of both tests simultaneously for the best possible demonstration of individual cases.

C.F. may be used advantageously for the diagnosis of animal brucellosis apparently with better results than those obtained in the human disease. According to the experiments of Gargani and Aleandri (1959), it is better to use the antigen of *Br. abortus* for the diagnosis of cattle brucellosis and to use the antigen of *Br. melitensis* for ovine brucellosis. Munkor (1958) examined 478 samples of cattle serum with C.F. and slow thorough agglutination for comparison purposes; he

observed more positive cases with sero-agglutination (205) than with cold frozen C.F. (164).

In accordance with the experiments of Lambert and Amerault (1962), the test is effective in the identification of the disease in noninfected cattle; but it is as sensitive as sero-agglutination with heated or unheated serum.

Alton and Jones (1963) give us a very complete description of C.F. which could be used as a standardized test for the diagnosis of brucellosis in animals. It was used primarily in cattle as a way of distinguishing vaccinated animals from naturally infected animals.

(c) Other Diagnostic Tests

Some other serological tests have been used sporadically in the diagnosis of human and animal brucellosis. The results, in general, are debatable; in some cases, further experimental and comparative study is necessary.

Among the laboratory tests which have not found acceptance and which possibly will not be accepted we have hemagglutination (Beert, 1958), the bactericidal capacity of blood (Minoprio, 1955; Minoprio and Harris, ...1955), the flocculation test (Hunter and Colbert, 1956; Gargani, et al, 1959; and the Goombs sic test, as well as Alivisatos et al, 1958; Cedro et al, 1959). These tests require standardization and will be used in special cases as a supplementary aid in diagnosis.

We have three tests which will have to be examined most carefully because they are very promising, not only in the diagnosis of individual cases but above all as research and investigation tests for the use among large population groups; they are: surface fixation, fluorescent antibodies, and "label" card tests.

Surface Fixation

This test was described 13 years ago by Castaneda (1950). We already have some publications available on it.

Although the test requires further standardization, not only as regards the preparation of the antigen but also as regards the technique and interpretation of the results, the advantages and simplicity are so outstanding as to justify further thorough study and comparison. Castaneda et al (1959) and Etchegaray and Pinel (1962) stated that there is a correlation between surface fixation and sero-agglutination, when

the tests were performed with cattle and hog sera, respectively. In man, Castaneda (1960) encountered 0.83% reactors [reagents] in a test of approximately 4,000 blood samples from blood donors in Mexico; on the other hand, Roldan (1963) encountered very few reactors with the surface fixation test and the sero-agglutination test; this involved 3,385 sera which were examined in a settlement in El Salvador where cattle brucellosis is enzootic. Table I summarizes the results obtained by the abovementioned investigators. We can note that the surface fixation test consistently gave more positive results than sero-agglutination.

Castaneda and associates (1959) interpreted this discrepancy as an attribute of the sensitivity of the test or its capacity to single out [indicate] more infected animals than other [tests].

Fluorescent Antibodies

This test is relatively new in the diagnosis of brucellosis and was tried with great success during an epidemic of brucellosis in a [meat] packing plant in Iowa in the United States (Biegeleisen, Jr. and associates, 1962, a, b). The indirect fluorescent antibody test was compared to the sero-agglutination test, showing up brucella antibodies in 96 human sera; the results show that the fluorescent antibodies can be seen even when the sero-agglutination test would have shown up very low titers. The direct fluorescent antibody test was positive in the tissues of hogs out of 109 treated [sic; in the tissue of 109 treated hogs]. The test will have to be studied more thoroughly and this should make it more useful in specialized laboratories.

"Label" /Tag/ Test

The basic principle behind this new test is so simple that it is astonishing that it has not been adopted long ago for the diagnosis of brucellosis. It is similar to the plate method, using blood plasma and a "label" [card, tag]; in order to obtain the plasma, we cover another card with dry anti-coagulant and phytoagglutinin. As recommended by Brewer et al (Warner, 1963) and as presented right now, the test is not very advisable because of the time it consumes.

Norrung (1955) many years ago described a similar test. He used cards on which he deposited a dry antigen stained with tetrazolium. A modification of the Norrung method is the method in which we use a drop of whole blood, as described by several investigators and mentioned at the beginning of this test; this will be a good exploration method.

Further experiments will have to be conducted, however; this would certainly be very helpful.

Milk Tests

No new changes have been worked out in the past several years in the various tests using milk or serum for the diagnosis of animal brucellosis. The ring test continues to be the best for the routine diagnosis of milk cattle brucellosis (Janny and Berman, 1959). Antigens stained with tetrazolium appear to be more sensitive than those stained with hematoxylin; this is perhaps due to the fact that, considering the handling of microorganisms with tetrazolium, we do not damage the cell wall since we are only staining the intracellular portions in which there is dehydrogenase activity (Mello et al, 1957).

Allergy Tests

The importance of this procedure for the diagnosis of brucellosis should not be overlooked. However the same recommendations, made at the last meeting of the committee of experts on brucellosis of the FAO/WHO (1958) should be kept in mind here. A positive reaction indicates prior exposition of to brucella but this does not necessarily signify the present infection of the subject.

Since different antigens have been used for making this test, it would be highly recommendable to undertake a large-scale antigen standardization program and to make comparisons with other tests. The rather wise comments of Castaneda (1961 b) should be taken into consideration in this connection.

For this diagnosis of the disease in humans, the extract of watery MBP, originally prepared by Castaneda and Carrillo (1941) for the treatment of brucellosis, is one of the most widely used allergens (see also Gabrielli and Caturegli, 1959), as well as the different watery extracts obtained by other investigators, such as the polysaccharides extracted by Leon and Cano (1958). The interpretation of the allergy test is a critical part of the entire operation here and should include a very careful examination of any local or general reaction (Parnas, 1957).

This test has been used quite a lot in animals, especially by Russian investigators, in the diagnosis of caprine goat brucellosis (Hoptman, 1959; Ivan sig, 1962).

Final Comments

The major portion of the problems encountered in the

past in the diagnosis of brucellosis in man and animals are still with us today. We can list them as follows:

(a) Absence of standardization of antigens and procedures.

(b) Insufficient number of comparative tests related to clinical evidence.

We might make two basic suggestions here:

(1) Research dealing with the abovementioned two points should be promoted;

(2) We should as soon as possible have published a monograph which should contain the presently satisfactory methods that are reliable; these should of course be methods for the diagnosis of brucellosis in man and other animals. This publication should be widely disseminated and might be something like the well-known publication "Rabies Laboratory Techniques" published by the WHO in the Monograph Series.

TABLE I

PERCENTAGE OF SURFACE FIXATION TESTS AND SERO-AGGLUTINATION TESTS IN BRUCELLOSIS IN MAN AND OTHER ANIMALS

a	b	c	d	e Seroagglutination	
				f	g
Especie	Autor	Num. de muestras	Fixacion superficial	Metodo en tubo	Metodo rapido en placa
h	Castaneda y al. 1959	350	0	0	
		424	17.7	9.7	
		201	22.4	18.4	
		5097	33.0	24.9	
i	Fitchgany & Pouch, 1962.	225	10.6	2.2	4.9
		225	6.4	3.1	3.1
j	Castaneda	1 (Caden-Ledes)			
j	Roblin, 1963.	4000	0.5		
		3387	0.1		0.1
		1 (Cakotakas)			

Legend: a -- species; b -- author; c -- number of samples; d -- surface fixation; e -- sero-agglutination; f -- test tube method; g -- rapid plate method /slide method/; h -- cattle; i -- hogs; j -- man; l -- heated.